AD_____

AWARD NUMBER: W81XWH-05-1-0262

TITLE: Role of CDK4 in Breast Development and Cancer

PRINCIPAL INVESTIGATOR: Haritha Reddy

CONTRACTING ORGANIZATION: Temple University
Philadelphia, Pennsylvania 19122-6024

REPORT DATE: April 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
April 2006	Annual Summary	10 Mar 05 – 9 Mar 06

Role of CDK4 in Breast Development and Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0262

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Haritha Reddy

E-mail: harithad@temple.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Temple University
Philadelphia, Pennsylvania 19122-6024

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Cdk4 is an important regulator of G1/S cell cycle progression in mammalian cells. In about 15.8% (15 out of 95) of breast cancers, cdk4 gene was shown to be amplified and this amplification of cdk4 gene was correlated with high Cdk4 protein expression. Our studies with the breast tissues of cdk4 (neo/neo) mice revealed the presence of small fat pads and poor ductal branching when compared to that of wild type mice. In order to determine the importance of cdk4 in Wnt- and Neu-induced breast tumorigenesis, we generated cdk4 (neo/neo): MMTV- transgenic lines that express Wnt and Neu in breast specific manner. Our results from these studies indicated that there is impaired lobuloalveolar compartment development and poor ductal branching in case of cdk4 (neo/neo): MMTV-neu mice when compared to cdk4 (+/+): MMTV-neu mice. In contrast, the mammary gland development in case of both Wnt transgenic mice, cdk4 (+/+): MMTV-Wnt and cdk4 (neo/neo): MMTV-Wnt, is comparable. Further studies revealed that there is resistance to neu-induced breast tumorogenesis in case of cdk4 (neo/neo): MMTV-neu mice when compared to cdk4 (+/+): MMTV-neu mice. In contrast, in case of Wnt transgenic mice, the tumorogenesis studies revealed that both cdk4 (+/+): MMTV-Wnt and cdk4 (neo/neo): MMTV-Wnt mice are equally susceptible to breast cancer induced by Wnt. This indicates that cdk4 is essential for neu-induced tumorigenesis and not for Wnt-induced tumorigenesis.

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)

CDK4, Breast Development, Oncogenes, Cell Cycle

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction:

Breast cancer is the most malignant cancer among women. In about 20% of human breast cancers, the *cyclin D1* gene was found to be amplified and overexpression of the Cyclin D1 protein was observed in about 50% of human breast cancers (1). These D type cyclins associate with CDK4, an important regulator of G1/S cell cycle progression of mammalian cells in culture. In about 15.8% (15 out of 95) of breast cancers, cdk4 gene was shown to be amplified and this amplification of cdk4 gene was correlated with high CDK4 protein expression (2). The histopathological analysis of breast tissue from cdk4 *neo/neo* mice revealed the presence of small fat pads and poor ductal branching when compared to that of wild type mice. This is in contrast to *cyclin D1 (-/-)* mice in which mammary gland development is comparable to wild type mice but show impairment of lobuloalveolar development during the late stages of pregnancy. These *cyclin D1 (-/-)* mice were resistant to mammary cancer driven by *neu* and *ras* protooncogenes but susceptible to those driven by *Wnt* and *myc* protooncogenes (1). It is not known if Cdk4 is required for these oncogenes to induce mammary tumors. Inorder to delineate the mechanisms associated with mammary tumor induction by these oncogenes, it is important to know if Cdk4 is required for the tumor induction by these oncogenes.

Body:

cdk4/ MMTV-*neu* and *cdk4*/ MMTV-*Wnt* transgenic mice:

We crossed *cdk4 (+/neo)* mice to MMTV-*neu* and MMTV-*Wnt* transgenic mice and generated *cdk4 (+/+)*: MMTV-*neu*, *cdk4 (neo/neo)*: MMTV-*neu*, *cdk4 (+/+)*: MMTV-*Wnt* and *cdk4 (neo/neo)*: MMTV-*Wnt* transgenic mice. With these mice we carried out histopathological analysis, tumor frequency studies and cell cycle protein analysis.

The histopathological studies of *cdk4 (+/+)*: MMTV-*neu* mice revealed the presence of proliferative disturbances in the mammary epithelium as evidenced by the appearance of hyperneoplastic nodules. In contrast to this, *cdk4 (neo/neo)*: MMTV-*neu* mice showed poor ductal branching and less lobuloalveolar development when compared to that of the wild type counterparts. Whereas in case of both *cdk4 (+/+)*: MMTV-*Wnt* and *cdk4 (neo/neo)*: MMTV-*Wnt* transgenic mice, the histopathological analysis of mammary glands showed robust development of lobuloalveolar compartment which is comparable to that of cdk4+/+ pregnant females.

The tumor frequency studies revealed the development of breast cancer in about ~97% of *cdk4 (+/+)*: MMTV-*neu* females at an age on 28 to 75 weeks, which is in contrast to *cdk4 (neo/neo)*: MMTV-*neu* females. In case of *cdk4 (neo/neo)*: MMTV-*neu* females, only 14% developed breast cancer by 60 weeks of age. Whereas in case of *Wnt* transgenic mice, >90% of mice developed breast cancer by 30 weeks of age.

The analysis of cell cycle proteins CDK6 and CDK2 showed that their expression is similar in the mammary tissues of all these transgenic mice.

The only technical difficulty encountered was regarding the standardization of kinase assays. We performed kinase assays to determine the activity of different cyclin dependent kinases and found nonspecific activity in these assays. Presently, we are trying to solve this problem using different methods.

cdk4/ MMTV-H-*ras* and *cdk4*/ MMTV-c-*myc* transgenic mice:

We are crossing *cdk4+/-* mice to MMTV-*ras* and MMTV-*Wnt* mice to generate statistically significant numbers for the tumorigenesis studies.

Key Research Accomplishments:

- Impaired mammary gland development in *cdk4 (neo/neo)*: MMTV-*neu* mice when compared to wild type counterparts
- Resistance to mammary tumors induced by *neu* oncogene in case of *cdk4 (neo/neo)*: MMTV-*neu* mice when compared to wild type counterparts
- Robust development of mmammary glands in case of *Wnt* transgenic mice irrespective of the presence or absence of *cdk4*
- Equal susceptibility of *Wnt* transgenic mice to mammary tumors irrespective of the presence or absence of *cdk4*

Reportable Outcomes:

Cyclin-Dependent Kinase 4 Expression is Essential for Neu Induced Breast Tumorigenesis
Haritha K.D.L. Reddy, Richard V. Mettus, Sushil G. Rane, Xavier Grana, Judith Litvin, and E. Premkumar Reddy
Cancer Res 2005; 65: (22). November 15, 2005

Conclusion:

Our results indicate that cdk4 is essential for neu-induced tumorigenesis and not for Wnt induced tumorigenesis. The presence of significantly similar levels of Cdk6 and Cdk2 shows that the loss of Cdk4 is not compensated by either Cdk6 or Cdk2.

References:

1. Q. Yu, Y. Geng, P. Sicinski, Nature 411, 1017 (2001).
2. H-X. An, M. W. Beckmann, G. Reifenberger, H. G. Bender, D. Niederacher, Am. J. Pathol. 154, 113 (1999).
3. S. G. Ran, P. Dubus, R. V. Mettus, E. J. Galbreath, G. Boden, E. P. Reddy, M. Barbacid, Nat. Genetics 22, 44 (1999).

Cyclin-Dependent Kinase 4 Expression Is Essential for Neu-Induced Breast Tumorigenesis

Haritha K.D.L. Reddy, Richard V. Mettus, Sushil G. Rane, Xavier Graña, Judith Litvin, and E. Premkumar Reddy

Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania

Abstract

Previous work has shown that cyclin D1 expression is required for *neu*- and *ras*-induced, but not *wnt*- or c-*myc*–induced, breast tumorigenesis in mice. Although cyclin D1 binds and activates cyclin-dependent kinase 4 (Cdk4), thereby mediating activation of a program of E2F-dependent gene expression, it has been suggested that the oncogenic activities of cyclin D1 are independent of Cdk4. To determine whether Cdk4 expression is required for breast tumorigenesis in mice, we have generated compound mice ectopically expressing the *neu* or *wnt* oncogenes in the mammary glands of wild-type and *Cdk4*−/− mice. Our results show that Cdk4 expression is required for efficient *neu*-induced tumorigenesis but is dispensable for *wnt*-induced breast tumorigenesis. In contrast to results previously observed in the mammary glands of *cyclin D1*−/− virgin females, our results show defects in mammary gland development in *Cdk4*−/− virgin females, suggesting differences in compensatory mechanisms in the absence of either subunit of the cyclin D1/Cdk4 complex. These results suggest that drugs targeted to inhibit Cdk4 activities could be developed to specifically treat certain breast tumors as Cdk4 is not essential for viability. (Cancer Res 2005; 65(22): 10174-8)

Introduction

A key response to growth factors in many cell types is the activation of cyclin-dependent kinase (Cdk) 4 or Cdk6 by members of the cyclin D family (D1, D2, and D3). D-type cyclins are expressed at low levels in a variety of quiescent cell types and their expression is stimulated by growth factors and mitogens (1–5). Approximately 50% of human mammary carcinomas express abnormally high levels of cyclin D1 (6–10), which is maintained throughout subsequent stages of breast cancer progression from *in situ* carcinoma to invasive carcinomas (9, 11, 12). Consistent with the oncogenic role of cyclin D1 in mammary epithelium, transgenic mice overexpressing cyclin D1 in their breast tissue have been found to develop mammary adenocarcinomas (13). Furthermore, loss of cyclin D1 was found to affect breast development (14, 15). More importantly, cyclin D1 null mutant mice were found to be resistant to breast cancers induced by the *neu* and *ras* oncogenes but remained fully sensitive to other oncogenic pathways driven by c-Myc or Wnt-1 (16). A requirement for D-type cyclins in cellular transformation *in vitro* has also been shown using triple *cyclin D* knockout mouse embryonic fibroblasts, which are resistant to transformation by c-Myc or Ras in combination with dn-p53, E1A, or c-Myc (17). Similarly, *Cdk4* null mouse embryonic fibroblasts have been shown to be refractory to transformation by Ras and dn-p53 and, consistent with these data, the hyperactive *Cdk4R24C* allele cooperates with single oncogenes to transform mouse embryonic fibroblasts *in vitro* (18, 19). Taken together, these results suggest that the activity of D-type cyclin/Cdk4 complexes is required for fibroblast transformation. However, it has also been suggested that the oncogenic function of cyclin D1 is independent of its ability to activate Cdks and is perhaps linked to the direct effects of cyclin D1 in controlling the expression of a subset of genes that are co-up-regulated in human tumors with deregulated cyclin D1 (20).

Thus, whereas a role for cyclin D1 in breast cancer is well established, it is not known whether the oncogenic function of cyclin D1 requires Cdk4. To understand the role of Cdk4 *in vivo*, we have targeted the mouse *Cdk4* locus by homologous recombination in embryonic stem cells and generated a strain of mice that does not express *Cdk4* [*Cdk4(neo/neo)*; ref. 21]. Homozygous *Cdk4(neo/neo)* null mutant mice are viable and were found to be very resistant to carcinogen-induced cancers (data not shown). In this communication, we show that loss of *Cdk4* expression results in poor mammary gland development that is characterized by impaired ductal branching. In addition, we show that Cdk4 expression is essential for *neu*-induced breast tumor development; on the other hand, it is dispensable for *wnt*-induced breast tumor development.

Materials and Methods

Generation of *Cdk4(neo/neo)*/mouse mammary tumor virus transgenic mice. To generate compound mice that express *neu* and *wnt*-1 oncogenes in a Cdk4 null background, *Cdk4(neo/+)* mice were mated with mouse mammary tumor virus (MMTV)-*neu* and MMTV-*wnt*-1 transgenic mice to generate *Cdk4(neo/+)*/MMTV-*neu* and *Cdk4(neo/+)*/MMTV-*wnt*-1 mice, respectively. These mice were then intracrossed to generate *Cdk4(neo/neo)*/MMTV-*neu* and *Cdk4(neo/neo)*/MMTV-*wnt*-1 transgenic mice.

Whole-mount and histopathologic analysis of mammary glands. The fourth inguinal mammary glands were dissected, spread onto a glass slide, and fixed with a mixture (1:3) of glacial acetic acid/ethanol, hydrated, stained with 0.2% carmine and 0.5% $AlK(SO_4)_2$, dehydrated in graded solutions of ethanol, and cleared in toluene and methyl salicylate as described previously (14). Carmine-stained or formalin-fixed mammary glands were also routinely processed for paraffin embedding and were stained with H&E.

Protein analysis. Mammary glands or tumors were homogenized in TNE lysis buffer and lysates were cleared by centrifugation. Protein, 50 to 100 μg, was resolved by SDS-PAGE and was transferred to nitrocellulose membranes. Immunoblots were probed with antibodies against HER2/ErbB2 (Cell Signaling Technology, Beverly, MA), Cdk4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Cdk6 (NeoMarkers, Fremont, CA), Cdk2 (Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam, Cambridge, MA), retinoblastoma (Rb; BD Biosciences, San Diego, CA), and phosphorylated Rb (pRb; Ser^{780}; Cell Signaling Technology).

Note: S.G. Rane is currently at Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, Bethesda, MD 20892.

Requests for reprints: E. Premkumar Reddy, Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Pharmacy Building, 3307 North Broad Street, Philadelphia, PA 19140. Phone: 215-707-4307; Fax: 215-707-1454; E-mail: reddy@temple.edu.

doi:10.1158/0008-5472.CAN-05-2639

Results

Cdk4 is required for proper development of mammary epithelium. To gain an insight into the role of Cdk4 in breast development, we first examined the status of mammary epithelium in wild-type [*Cdk4(+/+)*] and *Cdk4*-deficient [*Cdk4(neo/neo)*] mice. Examination of H&E-stained mammary gland whole mounts derived from virgin female mammary glands at 14 to 17 weeks revealed striking differences in the extent of mammary gland ductal outgrowth in the two sets of mice (Fig. 1*A* and *C*). In *Cdk4(neo/neo)* mice, both ductal outgrowth and branching morphogenesis was considerably reduced when compared with their wild-type counterparts. In addition, an examination of the longitudinal sections of the mammary tissue sections also showed a distinctive reduction in the number of mammary ducts and a complete absence of alveoli (Fig. 1*B* and *D*). These observations suggest that loss of Cdk4 expression in breast epithelium results in a diminution of mammary gland ductal branching where alveolar segments were markedly fewer in number compared with the wild-type mammary gland.

Effect of loss of Cdk4 expression on *neu*- and *wnt*-mediated breast tumorigenesis. To study the role of Cdk4 in *neu*- and *wnt*-induced breast tumorigenesis, *Cdk4(neo/neo)* mice were bred with MMTV-*neu* and MMTV-*wnt* transgenic mice to generate *Cdk4(neo/neo)*:MMTV-*neu*, and *Cdk4(neo/neo)*:MMTV-*wnt* mice, respectively (Fig. 2*A*). Whole-mount and histopathologic sections of the mammary glands derived from virgin adult mice (~14 weeks) from these different crosses (Fig. 2*B* and *F*) showed that *Cdk4(+/+)*:MMTV-*neu* mice exhibit proliferative disturbances in the mammary epithelium as evidenced by the appearance of multiple hyperplastic and dysplastic nodules that infiltrate the mammary fat pad (Fig. 2*B* and *F*), which is in accordance with the published data (22). Similar examination of whole-mount and histopathologic sections of mammary tissue derived from *Cdk4(neo/neo)*:MMTV-*neu* mice showed that the ductal outgrowth and branching morphogenesis was considerably reduced compared with *Cdk(+/+)*:MMTV-*neu* mice with distinctive absence of any hyperplastic or dysplastic nodules that are characteristic of the latter group of mice (Fig. 2*C* and *G*). Histopathologic examination of these mammary glands also failed to show abnormal proliferative disturbances in the mammary epithelium of *Cdk4(neo/neo)*:MMTV-neu mice (Fig. 2*C* and *G*). This does not seem to be due to lack of Neu expression, as equal levels of Neu protein was seen in both *Cdk4(+/+)*:MMTV-*neu* and *Cdk4(neo/neo)*:MMTV-*neu* mice (Fig. 2*J*). These results suggest that Cdk4 expression is essential for the appearance of MMTV-*neu*–induced proliferative disturbances that are seen in *Cdk4(+/+)*:MMTV-*neu* mice.

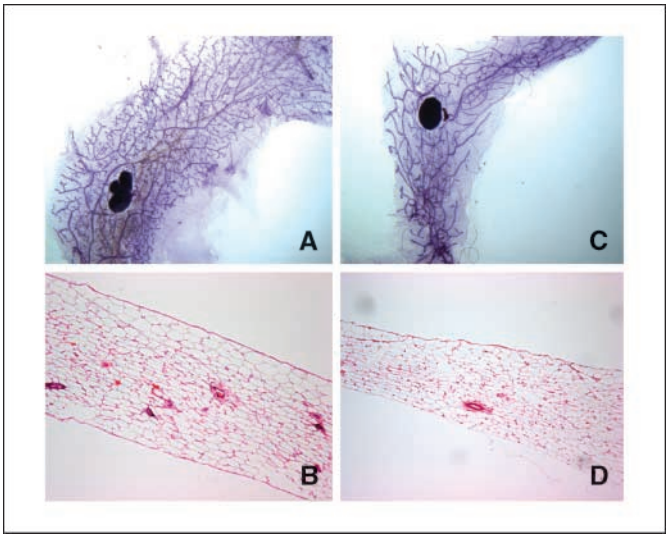

Figure 1. Impaired mammary epithelial expansion in *Cdk4(neo/neo)* mice. The fourth inguinal mammary glands from *Cdk4(+/+)* (*A*) and *Cdk4(neo/neo)* (*C*) mice at 14 weeks of age were removed, fixed, and stained with carmine alum stain overnight at room temperature. Histologic sections of the fourth inguinal mammary glands from *Cdk4(+/+)* mice (*B*) and *Cdk4(neo/neo)* mice (*D*) were stained with H&E.

In contrast to MMTV-*neu* mice, the mammary glands of virgin *Cdk4(+/+)*:MMTV-*wnt* mice showed precocious lobuloalvelar development that resembles that of *Cdk4(+/+)* pregnant female mice (Fig. 2*D* and *H*), similar to previously reported observations (23). Histopathologic examination of these mammary glands revealed extensive appearance of hyperplastic alveolar nodules, which seem to be preneoplastic lesions (23). Similar examination of whole-mount and histopathologic sections of mammary tissue derived from *Cdk4(neo/neo)*:MMTV-*wnt* mice showed that the ductal outgrowth and branching morphogenesis was unaltered compared with *Cdk(+/+)*:MMTV-*wnt* mice (Fig. 2*E* and *I*). Histopathologic examination of these mammary tissues again showed extensive appearance of hyperplastic alveolar nodules, similar to that seen with wild-type MMTV-*wnt* mice. These results indicate that MMTV-*wnt*–induced proliferative disturbances do not require Cdk4 expression.

Loss of expression of Cdk4 influences the incidence of mammary carcinomas. It has been previously reported that MMTV-*neu*–induced breast carcinomas require the expression of cyclin D1, whereas those induced by MMTV-*wnt* do not require the expression of cyclin D1 (16). To determine whether Cdk4 plays a similar role in the development of breast carcinomas, we monitored the four groups of transgenic mice for the appearance of breast tumors. The results of this study presented in Fig. 3*A* show that ~97% of the *Cdk4(+/+)*:MMTV-*neu* mice develop breast cancer between 28 to 75 weeks of age. The rest of the mice were found to develop salivary gland tumors. In sharp contrast, only ~14% of the *Cdk4(neo/neo)*:MMTV-*neu* mice develop signs of breast cancer and this incidence was found to occur only after ~60 weeks of age; when these tumors arise, they were very small in size compared with their wild-type counterparts. Calculation of *P* values showed a highly significant increase in tumor frequency ($P = 2.3 \times 10^{-6}$) for *Cdk4(+/+)*:MMTV-*neu* mice as opposed to their *neo/neo* counterparts. These observations suggest that development of breast tumors in MMTV-*neu* transgenic mice requires normal expression of Cdk4.

In contrast to *Cdk4(+/+)*-MMTV-*neu* mice, both *Cdk4(+/+)*:MMTV-*wnt* mice and *Cdk4(neo/neo)*:MMTV-*wnt* mice exhibited a rapid onset of breast tumors around 10 weeks of age and >90% of these mice developed breast tumors by the age of 30 weeks (Fig. 3*B*). Our studies also show that there was a slight delay in the development of breast tumors in *Cdk4(neo/neo)*:MMTV-*wnt* mice compared with their wild-type counterparts. In contrast to the results observed for MMTV-*neu* mice, no significant difference in tumor frequency ($P = 0.7264$) was observed between *Cdk4(+/+)*:MMTV-*wnt*-1 and their *neo/neo* counterparts. The incidence of breast tumors was seen in both male and female mice as has been previously described (22). These observations show that Cdk4 expression is dispensable for MMTV-*wnt*–induced breast tumorigenesis.

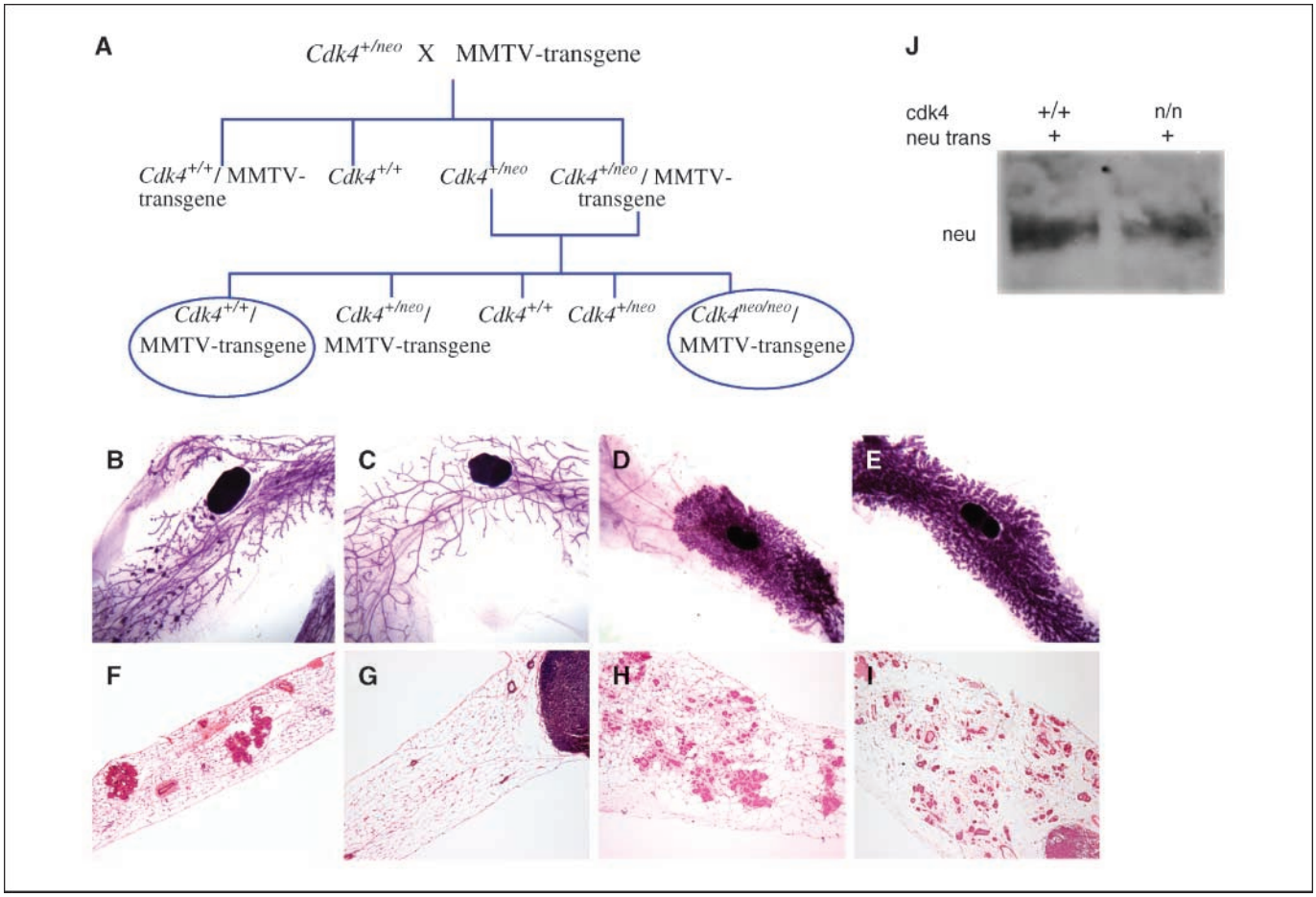

Figure 2. Loss of Cdk4 impairs MMTV-neu–induced breast epithelial cell proliferation and formation of preneoplastic nodules but not of the MMTV-*wnt*-1–induced transformation. *A,* crosses done to produce the required transgenic mice. Whole mounts were made from the fourth inguinal mammary glands of *Cdk4(+/+)*/MMTV-*neu* (*B*), *Cdk4(neo/neo)*/MMTV-*neu* (*C*), *Cdk4(+/+)*/MMTV-*wnt*-1 (*D*), and *Cdk4(neo/neo)*/MMTV-*wnt*-1 (*E*) transgenic mice. *F, G, H*, and *I,* H&E-stained sections of the mammary glands shown in *B, C, D*, and *E* respectively. *J,* Western blot analysis of mammary tissue extracts derived from *Cdk4(neo/neo)* and *Cdk4(+/+)* mice. Each lane contains 100 μg of protein. Western blot analysis was done with an anti-neu antibody.

The histopathologic sections of the tumors is given in Fig. 3*C*. These sections show that *Cdk4(+/+)*:MMTV-*neu* tumors have a high density of epithelial cells, whereas the tumor sections of *Cdk4(neo/neo):*MMTV-*neu* mice show increased infiltration by connective tissue. MMTV-*wnt* tumors in a *Cdk4(+/+)* or *Cdk4(neo/neo)* background showed a similar phenotype with a high density of epithelial cells. Interestingly, these tumors show increased vasculature, suggesting that the Wnt pathway promotes angiogenesis, which might explain the very rapid growth of tumors in these mice.

Expression patterns of Cdk4, Cdk6, and Cdk2. It has been previously shown that loss of *cyclin* D1 results in a breast tumor phenotype similar to that described here for *Cdk4 (neo/neo)* mice. In the case of MMTV-*wnt*:cyclin D−/− mice, loss of *cyclin D1* seemed to be compensated by the overexpression of cyclin D2, which could drive the cell cycle progression (16). To understand the molecular basis for the development of breast tumors in MMTV-*wnt* mice in a *Cdk4* null background, we examined the expression patterns and kinase activities of Cdk6 and Cdk2 in all four of the genotypes studied here. The results presented in Fig. 4*A* show that in both *Cdk4(neo/neo):*MMTV-*wnt* and in *Cdk4(neo/neo):*MMTV-*neu* mice, the levels of Cdk4 were undetectable, whereas the levels of Cdk4 were pronounced in *Cdk4(+/+):*MMTV-*neu* and *Cdk4(+/+):* MMTV-*wnt* mice. In contrast, the levels of Cdk6 and Cdk2 were approximately equal in all four genotypes. These results suggest that neither Cdk6 nor Cdk2 compensate for the loss of Cdk4 in MMTV-*wnt* transgenic mice on a *Cdk4(neo/neo)* background. We next examined the expression levels and the phosphorylation status of Rb in *Cdk4(neo/neo), Cdk4(+/+)* as well as the MMTV-*neu* and MMTV-*wnt* transgenic mice crossed to the two Cdk4 backgrounds. Results of these experiments presented in Fig. 4*B* show that the level of pRb Ser^{780} phosphorylation was low in *Cdk(+/+)* tissues but showed considerable elevation in those derived from both *Cdk4(+/+):*MMTV-*neu* and Cdk4(+/+):MMTV-*wnt* mice, which corresponds to the high levels of Cdk4, Cdk6, and Cdk2 activities seen in these tissues.

Discussion

Our studies reported in this article suggest the importance of *Cdk4* in mammary gland development and tumorigenesis. Whole-mount analysis and histologic sections of *Cdk4(neo/neo)* and *Cdk4(+/+)* mice show that Cdk4 is required for proper ductal branching and lobuloalveolar development of virgin female mice. In contrast, the mammary glands of *cyclin D1−/−* virgin females have been reported to be identical to that of wild-type mice. This difference is likely due to the compensation by cyclins D2 and D3,

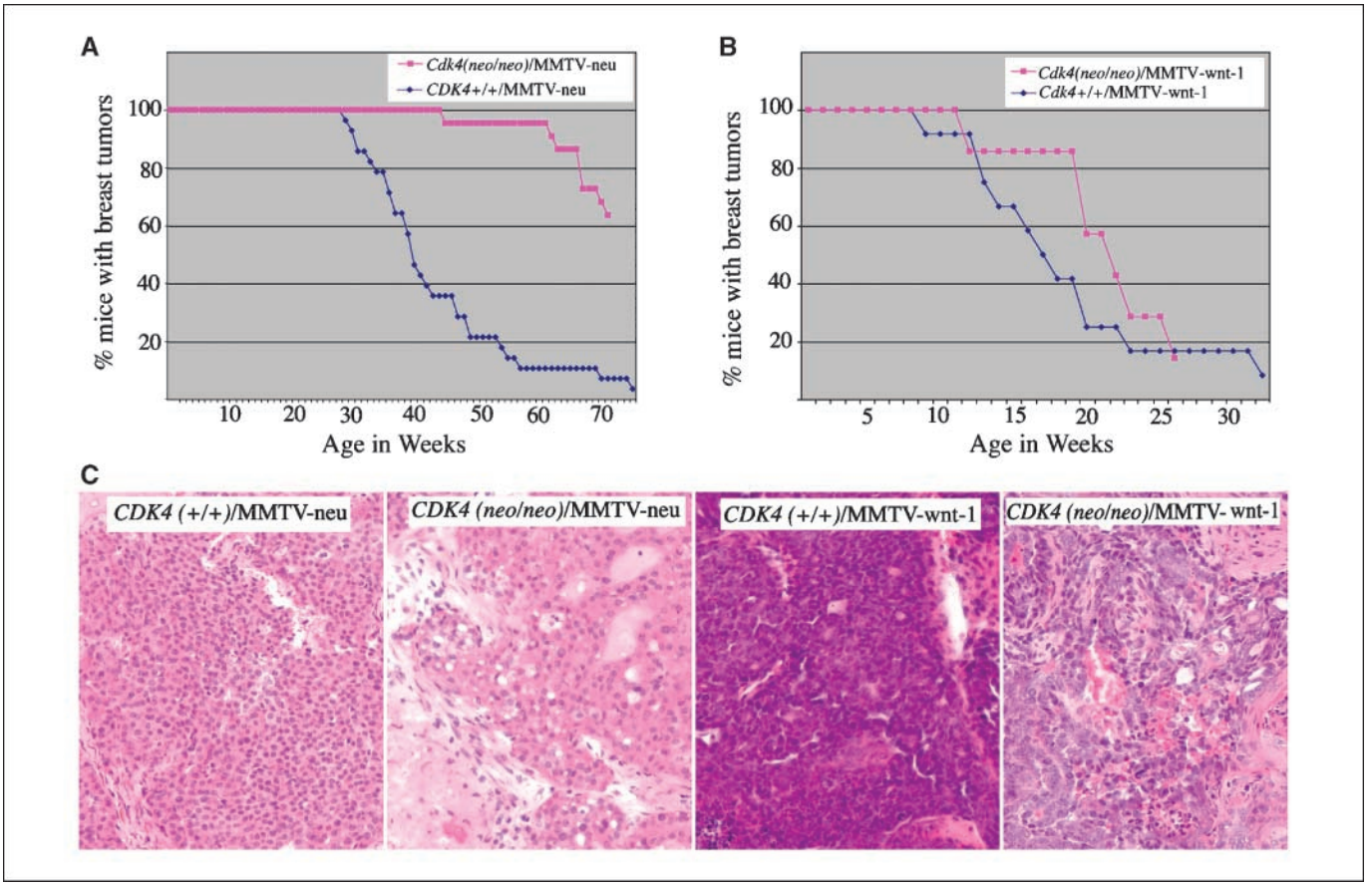

Figure 3. Loss of Cdk4 results in reduced and delayed tumor incidence in *Cdk4(neo/neo)*/MMTV-*neu* transgenic mice. *A,* tumor incidence in *Cdk4(neo/neo)*/MMTV-*neu* and *Cdk4(+/+)*/MMTV-*neu* mice over a period of 75 weeks. *B,* tumor incidence in *Cdk4(neo/neo)*/MMTV-*wnt*-1 and *Cdk4(+/+)*/MMTV-*wnt*-1 mice over a period of 31 weeks. *C,* histology of tumors in MMTV-*neu* and MMTV-*wnt* mice bred against Cdk4(+/+) or *Cdk4(neo/neo)* background. Tumor sections were stained with H&E and photographed at a magnification of ×100.

which are slightly up-regulated in the mammary gland of *cyclin D1−/−* virgin females (16). Our results suggest that in the absence of Cdk4, there is no parallel compensation by other Cdks (Fig. 4, see below). Regardless of the lack of defects in the mammary gland of *cyclin D1−/−* virgin females, it has been shown that *cyclin D1−/−* mice fail to undergo full lobuloalveolar development during late stages of pregnancy (14, 15). It has also been shown that the cyclin D1 null mice are prone to transformation induced by the *wnt*-1 and *myc* oncogenes, but not to transformation induced by the *neu* and *ras* oncogenes (16). Recent studies on MMTV-*erbB2*-MMTV-p16 double-transgenic mice showed that erb2-mediated tumorigenesis is blocked by p16 and that these double-transgenic mice develop rare tumors after a long delay (24). Because *Cdk4(neo/neo):*MMTV-*neu* mice showed decreased levels of ductal branching and lobuloalveolar development of the mammary glands when compared with that of *Cdk4(+/+):*MMTV-*neu* transgenic mice, we presume that Cdk4 is required for Neu-induced proliferative events that lead to ductal branching, lobuloalveolar development, and the development of hyperneoplastic alveolar nodules, and ultimately for the development of mammary tumors. We cannot rule out, however, that the observed defects in *Cdk4(neo/neo)* mammary gland development be the indirect result of hormonal signaling deficiencies as opposed to an epithelial cell autonomous defect.

The mammary glands of *Wnt-1* transgenic virgin mice undergo precocious lobuloalveolar development and resemble the mammary glands of wild-type nontransgenic pregnant females. Our whole-mount and histologic studies of the mammary glands of *Cdk4(neo/neo):*MMTV-*wnt*-1 and *Cdk4(+/+):*MMTV-*wnt*-1 mice showed comparable lobuloalveolar development. This suggests that Cdk4 is not required for Wnt-1–induced ductal branching and lobuloalveolar development. The tumorigenesis studies conducted by us also show that both strains of mice are equally susceptible to Wnt-1-induced tumorigenesis, suggesting that Cdk4 is not required for this process. Thus, if the defect in mammary development observed in *Cdk4 neo/neo* females is not cell autonomous, then Wnt not only bypasses Cdk4 function, but also any conceivable defects in hormone signaling resulting from Cdk4 ablation. Our data also showed that the level of pRb phosphorylation on Ser^{780} correlated with G_1 Cdk activities. We have also seen that phosphorylation of this site on pRb is highly increased in breast tumor tissues independently of Cdk4 phosphorylation status, suggesting that in highly proliferative tumors, this site could be phosphorylated by Cdks other that Cdk4 (data not shown).

Considering previous results indicating that Neu may act by inducing cyclin D1 expression and our results shown here that Cdk4 is required for *neu*-induced tumorigenesis, we propose that the cyclin D1/Cdk4 complex is required for *neu*-induced tumorigenesis. It has also been suggested that *wnt*- and c-*myc*-induced breast tumorigenesis communicate with the cell cycle machinery in breast epithelial cells through different targets. In this regard,

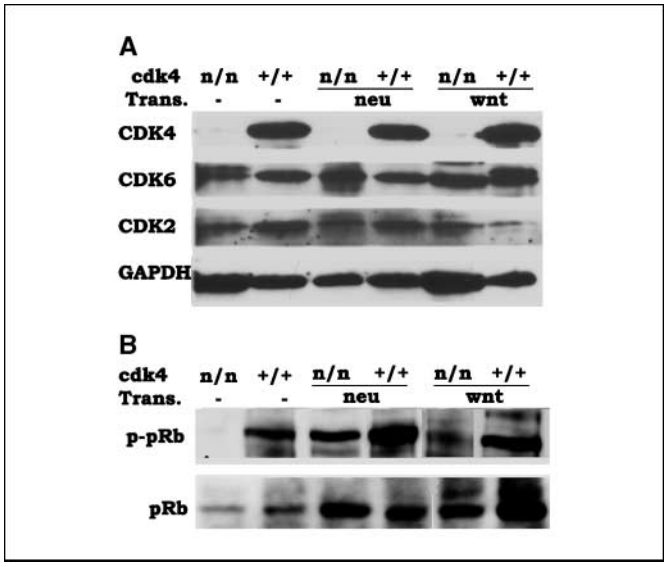

Figure 4. Cdk4, Cdk6, and Cdk2 expression and activity. *A,* Western blot analysis of the protein extracted from frozen mammary tissues of *Cdk4(neo/neo), Cdk4(+/+), Cdk4(neo/neo)*/MMTV-neu, *Cdk4(+/+)*/MMTV-*neu, Cdk4(neo/neo)*/MMTV-wnt-1, and *Cdk4(+/+)*/MMTV-wnt-1 transgenic mice using antibodies against Cdk4, Cdk6, Cdk2, and GAPDH (loading control). Each lane contains 50 μg of protein. *B,* expression of unphosphorylated Rb (*pRb*) and phosphorylated Rb (*p-pRb*) in mouse mammary extracts derived from (*A*). Each lane contains 50 μg of protein. *n/n, neo/neo*.

cyclin D2 expression was found to be up-regulated in tumors induced by *wnt*-1 and c-*myc*, but not *neu* or *ras* (16). Considering our data showing that Cdk4 is also dispensable for *Wnt*-induced tumorigenesis, and the lack of obvious compensation by other G_1 Cdks, it is tempting to speculate that Wnt signals downstream of D-type cyclin/Cdk4 complexes. In summary, our data suggest that, at least in the case of *Neu*-induced tumorigenesis, a Cdk4 function is required. This requirement could be for Cdk4 kinase activity, or, alternatively, for the ability of the cyclin D/Cdk4 complex to sequester p27. Further studies are necessary to differentiate between these two possibilities.

These results also have important implications with respect to therapeutic modalities that might be effective in the treatment of breast cancers that are neu-positive. The importance of Cdk4 and cyclin D1 complex in the genesis of *Neu*-induced breast tumors suggests that small molecule inhibitors of Cdk4 kinase activity could be very effective in blocking the growth of these human breast tumors, which often represent the most aggressive forms of human breast cancer.

Acknowledgments

Received 8/1/2005; revised 9/13/2005; accepted 9/21/2005.

Grant support: NIH P01 CA95569 and R01 AG22022.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Sherr CJ. The Pellicoller lecture: cancer cell cycle revisited. Cancer Res 2000;60:3689–95.
2. Grana X, Reddy EP. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (Cdks), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 1995;11:211–9.
3. Blagosklonny MV, Pardee AB. The restriction point of the cell cycle. Cell Cycle 2003;1:103–10.
4. Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 1997;13:261–91.
5. Malumbres A, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nat Cancer Rev 2001;1: 222–35.
6. Vuckley MF, Sweeney KJ, Hamilton JA, et al. Expression and amplification of cyclin genes in breast cancer. Oncogene 1993;8:2127–33.
7. Dickson C, Fantl V, Gillet C, et al. Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. Cancer Lett 1995;90:43–50.
8. Lammie GA, Fantl V, Smith R, et al. D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1 oncogene. Oncogene 1991;6:439–44.
9. Gillet C, Smith P, Gregory W, et al. Cyclin D1 and prognosis in human breast cancer. Int J Cancer 1996; 69:612–22.
10. McIntosh GG, Anderson JJ, Milton I, et al. Determination of the prognostic value of cyclin D1 overexpression in breast cancer. Oncogene 1995;11:885–91.
11. Bartkova J, Likas J, Strauss M, Bartek J. Cyclin D1 protein expression and function in human breast cancer. Int J Cancer 1994;57:353–61.
12. Weinstat-Saslow D, Merino MJ, Manrow RE, et al. Overexpression of cyclin D mRNA distinguishes invasive and *in situ* breast carcinomas from non-malignant lesions. Nat Med 1995;1:1257–60.
13. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt V. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature 1994;369:669–71.
14. Sicinski P, Donaher JL, Parker SB, et al. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell 1995;82:621–30.
15. Fantl V, Stamp G, Andrews A, Rosewell I, Dickson C. Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev 1995; 9:2364–72.
16. Yu Q, Geng Y, Sicinsky P. Specific protection against breast cancers by cyclin D1 ablation. Nature 2001;411: 1017–21.
17. Kozar K, Ciemerych MA, Rebel VI, et al. Mouse development and cell proliferation in the absence of D-cyclins. Cell 2004;118:477–91.
18. Zou X, Ray D, Aziyu A, et al. Cdk4 disruption renders primary mouse cells resistant to oncogenic transformation, leading to Arf/p53-independent senescence. Genes Dev 2002;16:2923–34.
19. Rane S, Cosenza S, Mettus RV, Reddy EP. Germline transmission of the Cdk4R24C mutation facilitates tumorigenesis and escape from cellular senescence. Mol Cell Biol 2002;22:644–56.
20. Lamb J, Ramaswamy S, Ford HL, et al. A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. Cell 2003; 114:323–34.
21. Rane SG, Dubus P, Mettus RV, et al. Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-islet cell hyperplasia. Nat Genet 1999;22:44–52.
22. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. Cell 1988;54:105–15.
23. Tsukamoto SA, Grosschedl R, Guzman RC, Parslow T, Varmus HE. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 1988;55:619–25.
24. Yang C, Ionescu-Tiba V, Burns K, et al. The role of the cyclin D1-dependent kinases in ErbB2-mediated breast cancer. Am J Pathol 2004;164:1031–8.